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(54) **METHOD AND MEDICAMENT FOR INHIBITING THE EXPRESSION OF A DEFINED GENE**

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- **MADHUR K. ET AL.: "Antisense RNA: function and fate of duplex RNA in cells of higher eukaryotes." MICROBIOLOGY AND MOLECULAR BIOLOGY REVIEWS, Vol. 62, December 1998 (1998-12), pages 1415-1434, XP000909741**

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Description

[0001] The invention relates to a method in accordance with the preamble of claim 1. It relates furthermore to a medicament according to the preamble of claim 32, to an active ingredient according to the preamble of claim 63, and to uses according to the preambles of claims 81 and 114.

[0002] A method of this type is known from WO 99/32619, which was unpublished at the priority date of the present invention. The known process aims to inhibit the expression of genes in cells of invertebrates. To this end, the double-stranded oligoribonucleotide must exhibit a sequence that is identical to the target gene and has a length of at least 25 bases. To achieve efficient inhibition, the identical sequence must be 300 to 1000 base pairs in length. Such an oligoribonucleotide is complicated to prepare.

[0003] The Unexamined German Patent Application No. DE 196 31 919 C2 describes an antisense RNA with specific secondary structures, the antisense RNA being present in the form of a vector encoding it. The antisense RNA is an RNA molecule complementary to regions of the mRNA. Inhibition of the gene expression is caused by binding to these regions. This inhibition can be employed in particular for the diagnosis and/or therapy of diseases, e.g., tumor diseases or viral infections. —The disadvantage is that the antisense RNA must be introduced into the cell in an amount that is at least as high as the amount of the mRNA. The known antisense methods are not particularly effective.

[0004] U.S. Pat. No. 5,712,257 discloses a medicament comprising mismatched double-stranded RNA (dsRNA) and bioactive mismatched fragments of dsRNA in the form of a ternary complex together with a surfactant. The dsRNA used for this purpose consists of synthetic nucleic acid single strands without a defined base sequence. The single strands undergo irregular base pairing, also known as "non-Watson-Crick" base pairing, giving rise to mismatched double strands. The known dsRNA is used to inhibit the replication of retroviruses such as HIV. Viral replication can be inhibited when non-sequence-specific dsRNA is introduced into the cells. This leads to induction of interferon, which is intended to inhibit viral replication. The inhibitory effect or the effectiveness of this method is poor.

[0005] It is known from Fire, A. et al., NATURE, Vol. 391, pp. 806, that dsRNA whose one strand is complementary in segments to a nematode gene to be inhibited inhibits the expression of this gene highly efficiently. It is believed that the particular effectiveness of the dsRNA used in nematode cells is not due to the antisense principle but possibly to the catalytic properties of the dsRNA, or enzymes induced by it. —Nothing is mentioned in this paper on the effectiveness of

specific dsRNA with regard to inhibiting gene expression, in particular in mammalian and human cells.

[0006] WO 92/19732 relates to antisense oligonucleotides and the use thereof. The single-stranded oligonucleotides are folded back or topologically closed to protect from enzymatic degradation by exonucleases. Their action is based in particular on blockade of target gene expression by triple helix bonds.

[0007] WO 98/05770 relates to an antisense RNA. The antisense RNA has a double-stranded region. This region is not complementary to the target gene, however. The double-stranded region is a self-complementary folding generated by a poly-GCGC ... sequence, which acts to stabilize the molecule.

[0008] Ulmann et al., 2377 Chemical Review, 90 (1990) June, No. 4, Washington DC, US provides an overview of the mode of action of antisense oligonucleotides.

[0009] The object of the present invention is to eliminate the disadvantages of the prior art. In particular, it is intended to provide the most effective method, medicament, or active ingredient possible and the most efficient use possible for the preparation of a medicament or active ingredient, which is capable of causing particularly effective inhibition of the expression of a defined target gene.

[0010] This object is achieved by the features of claims 1, 32, 63, 81, and 114. Advantageous embodiments can be seen from claims 2 to 31, 33 to 62, 882 [sic, 82?] to 113, and 115 to 125.

[0011] The object is achieved, inter alia, by the features of claim 1.

[0012] Surprisingly, it has emerged that an effective inhibition of target gene expression can be achieved even when the complementary region is less than 25 successive nucleotide pairs base pairs in length. The procedure of providing such oligoribonucleotides is less complicated.

[0013] In particular, dsRNA with a length of over 50 nucleotide pairs induces certain cellular mechanisms, e.g., dsRNA-dependent protein kinase or the 2-5A system, in mammalian and human cells. This leads to the disappearance of the interference effect mediated by dsRNA having a defined sequence. As a consequence, protein biosynthesis in the cell is blocked. The present invention overcomes this disadvantage in particular.

[0014] Furthermore, the uptake of dsRNA with short chain lengths into the cell or into the nucleus is facilitated markedly compared with longer-chain dsRNAs.

[0015] It has proved advantageous for the dsRNA or the vector to be present in micellar structures, preferably in liposomes. The dsRNA can likewise be enclosed in natural viral capsids or in chemically or enzymatically produced artificial capsids or structures derived therefrom. — The abovementioned features make it possible to introduce the dsRNA into defined target cells.

[0016] The gene to be inhibited is expediently expressed in eukaryotic cells. The target gene can be selected from the following group: oncogene, cytokine gene, Id protein gene, developmental gene, and prion gene. It can also be expressed in pathogenic organisms, preferably in plasmodia. It can be part of a virus or viroid which is preferably pathogenic to humans. —The method proposed makes it possible to produce agents for the treatment of genetically determined diseases, e.g., cancer, viral diseases, or Alzheimer's disease.

[0017] The virus or viroid can also be a virus or viroid that is pathogenic to animals or phytopathogenic. In this case, the method according to the invention also permits the provision of agents for treating animal or plant diseases.

[0018] According to a further aspect, the dsRNA is formed double-stranded in segments. The ends of the dsRNA can be modified to counteract degradation in the cell or dissociation into single strands. Dissociation takes place in particular when low concentrations or short chain lengths are used. To inhibit dissociation in a particularly effective fashion, the cohesion of the double-stranded structure can be increased by at least one, preferably two, further chemical linkage(s). —A dsRNA according to the invention whose dissociation is reduced exhibits greater stability to enzymatic and chemical degradation in the cell or in the organism.

[0019] The chemical linkage is expediently formed by a covalent or ionic bond, a hydrogen bond, hydrophobic interactions, preferably van-der-Waals or stacking interactions, or by metal-ion coordination. In an especially advantageous aspect, it can be formed at at least one, preferably both end(s).

[0020] It has furthermore proved to be advantageous for the chemical linkage to be formed by one or more linkage groups, the linkage groups preferably being poly(oxyphosphinicooxy-1,3-propanediol) and/or polyethylene glycol chains. The chemical linkage can also be formed by purine analogs used in place of purines in the double-stranded structure. It is advantageous furthermore for the chemical linkage to be formed by azabenzene units introduced into the double-stranded structure. Moreover, it can be formed by branched nucleotide analogs used in place of nucleotides in the double-stranded structure.

[0021] It has proved expedient to use at least one of the following groups for generating the chemical linkage: methylene blue; bifunctional groups, preferably bis(2-chloroethyl)amine; N-acetyl-N'-(p-glyoxylbenzoyl)cystamine; 4-thiouracil; and psoralene. The chemical linkage can furthermore be formed by thiophosphoryl groups attached at the ends of the double-stranded region. The chemical linkage at the ends of the double-stranded region is preferably formed by triple helix bonds.

[0022] The chemical linkage can expediently be induced by ultraviolet light.

[0023] The nucleotides of the dsRNA can be modified. This counteracts the activation, in the cell, of a double-stranded RNA-dependent protein kinase, PKR. Advantageously, at least one 2'-hydroxyl group of the dsRNA nucleotides in the double-stranded structure is replaced by a chemical group, preferably a 2'-amino or a 2'-methyl group. At least one nucleotide in at least one strand of the double-stranded structure can also be a locked nucleotide with a sugar ring which is chemically modified preferably by a 2'-O, 4'-C methylene bridge. Advantageously, several nucleotides are locked nucleotides.

[0024] A further especially advantageous embodiment provides that the dsRNA is bound to, associated with, or surrounded by at least one viral coat protein which originates from a virus, is derived therefrom, or has been prepared synthetically. The coat protein can be derived from polyomavirus. The coat protein can contain the polyomavirus virus protein 1 (VP1) and/or virus protein 2 (VP2). The use of such coat proteins is known from, e.g., Unexamined German Patent Application DE 196 18 797 A1. —The abovementioned features considerably facilitate the introduction of the dsRNA into the cell.

[0025] When a capsid or capsid-type structure is formed from the coat protein, one side preferably faces the interior of the capsid or capsid-type structure. The construct formed is particularly stable.

[0026] The dsRNA can be complementary to the primary or processed RNA transcript of the target gene. —The cell can be a vertebrate cell or a human cell.

[0027] At least two dsRNAs that differ from each other can be introduced into the cell, where at least segments of one strand of each dsRNA are complementary to in each case one of at least two different target genes. This makes it possible simultaneously to inhibit the expression of at least two different target genes. In order to suppress, in the cell, the expression of a double-stranded RNA-dependent protein kinase, PKR, one of the target genes is advantageously the PKR gene. This allows effective suppression of the PKR activity in the cell.

[0028] According to the invention, the object is achieved furthermore by a medicament with the features of claim 32 and/or 63. —Surprisingly, it has emerged that a dsRNA of this type is suitable as a medicament for inhibiting the expression of a defined gene in mammalian cells or in a phytopathogenic virus or viroid. In comparison with the use of single-stranded oligoribonucleotides, the inhibition is already caused at concentrations that are lower by at least one order of magnitude. The medicament according to the invention is highly effective. Lesser side effects can be expected.

[0029] According to the invention, furthermore, the object is achieved by the use of an oligoribonucleotide with the features of claim 81. —Surprisingly, a dsRNA of this type is suitable

for preparing a medicament for inhibiting the expression of a defined gene. Compared with the use of single-stranded oligoribonucleotides, the inhibition is already caused at concentrations that are lower by one order of magnitude when using dsRNA. The use according to the invention thus makes possible the preparation of particularly effective medicaments.

[0030] According to the invention, furthermore, the object is achieved by the use of a vector with the features of claim 114. —The use of a vector of this type makes possible a particularly effective gene therapy.

[0031] With regard to advantageous embodiments of the medicament and of the use, reference is made to the description of the aforementioned features.

[0032] Exemplary embodiments of the invention are illustrated in greater detail hereinbelow with reference to the figures, in which:

Figure 1 shows the schematic drawing of a plasmid for the *in vitro* transcription with T7 and SP6 polymerase,

Figure 2 shows RNA following electrophoresis on an 8% polyacrylamide gel and staining with ethidium bromide,

Figure 3 shows a representation of radioactive RNA transcripts following electrophoresis on an 8% polyacrylamide gel with 7 M urea by means of an instant imager, and

Figures 4a-e show Texas Red and YFP fluorescence in murine fibroblasts.

Exemplary Embodiment 1:

[0033] The inhibition of transcription was detected by means of dsRNA with sequence homology in an *in vitro* transcription system with a nuclear extract from human HeLa cells. The DNA template for this experiment was plasmid pCMV1200 which had been linearized by means of *Bam*HI.

Generation of Template Plasmids:

[0034] The plasmid shown in Fig. 1 was constructed for use in the enzymatic synthesis of dsRNA. To this end, a polymerase chain reaction (PCR) with the "positive control DNA" of the

HelaScribe® Nuclear Extract *in vitro* transcription kit from Promega, Madison, USA, as the DNA template was carried out first. One of the primers used contained the sequence of an *Eco*RI cleavage site and of the T7 RNA polymerase promoter as shown in sequence ID No. 1. The other primer contained the sequence of a *Bam*HI cleavage site and of the SP6 RNA polymerase promoter as shown in sequence ID No. 2. In addition, the two primers had, at the 3' ends, regions that were identical or complementary to the DNA template. The PCR was carried out by means of the "*Taq PCR Core Kit*" from Qiagen, Hilden, Germany, following the manufacturer's instructions. 1.5 mM $MgCl_2$, in each case 200 µM dNTP, in each case 0.5 µM primer, 2.5 U of *Taq* DNA polymerase, and approximately 100 ng of "positive control DNA" were employed as the template in PCR buffer in a volume of 100 µL. After initial denaturation of the template DNA by heating for 5 minutes at 94 °C, amplification was carried out in 30 cycles of denaturation for in each case 60 seconds at 94 °C, annealing for 60 seconds at 5 °C below the calculated melting point of the primers, and polymerization for 1.5–2 minutes at 72 °C After a final polymerization of 5 minutes at 72 °C, 5 µL of the reaction mixture was analyzed by agarose gel electrophoresis. The length of the DNA fragment amplified thus was 400 base pairs, 340 base pairs corresponding to the "positive control DNA." The PCR product was purified, hydrolyzed with *Eco*RI and *Bam*HI and, after repurification, employed for the ligation together with a pUC18 vector likewise hydrolyzed by *Eco*RI and *Bam*HI. *E. coli* XL1-blue was then transformed. The plasmid obtained (pCMV5) carries a DNA fragment whose 5' end is flanked by the T7 promoter and whose 3' end is flanked by the SP6 promoter. By linearizing the plasmid with *Bam*HI, it can be employed *in vitro* with the T7 RNA polymerase for the *run-off* transcription of a single-stranded RNA, which is 340 nucleotides in length and shown in sequence ID No. 3. If the plasmid is linearized with *Eco*RI, it can be employed for the *run-off* transcription with SP6 RNA polymerase, giving rise to the complementary strand. In accordance with the method outlined hereinabove, an RNA 23 nucleotides in length was also synthesized. To this end, a DNA shown in sequence ID No. 4 was ligated with the pUC18 vector via the *Eco*RI and *Bam*HI cleavage sites.

[0035] Plasmid pCMV1200 was constructed as the DNA template for the *in vitro* transcription with HeLa nuclear extract. To this end, a 1191 bp *Eco*RI/*Bam*HI fragment of the positive control DNA contained in the HeLaScribe® Nuclear Extract *in vitro* transcription kit was amplified by means of PCR. The amplified fragment comprises the 828 bp "immediate early" CMV promoter and a 363 bp transcribable DNA fragment. The PCR product was ligated to the vector pGEM-T via "T-overhang" ligation. There is a *Bam*HI cleavage site at the 5' end of the fragment. The

plasmid was linearized by hydrolysis with *Bam*HI and used as the template in the run-off transcription.

In vitro Transcription of the Complementary Single Strands:

[0036] pCMV5 plasmid DNA was linearized with *Eco*RI or *Bam*HI. It was used as the DNA template for an *in vitro* transcription of the complementary RNA single strands with SP6 and T7 RNA polymerase, respectively. The "Riboprobe *in vitro* Transcription" system from Promega, Madison, USA, was employed for this purpose. Following the manufacturer's instructions, 2 µg of linearized plasmid DNA was incubated in 100 µL of transcription buffer and 40 U T7 or SP6 RNA polymerase for 5–6 hours at 37 °C The DNA template was subsequently degraded by addition of 2.5 µL of RNase-free DNase RQ1 and incubation for 30 minutes at 37 °C The transcription mixture was made up to 300 µL with H_2O and purified by phenol extraction. The RNA was precipitated by addition of 150 µL of 7 M ammonium acetate and 1125 µL of ethanol and stored at -65 °C until used for hybridization.

Generation of the RNA Double Strands:

[0037] For the hybridization, 500 µL of the single-stranded RNA, stored in ethanol and precipitated, were spun down. The resulting pellet was dried and taken up in 30 µL of PIPES buffer, pH 6.4, in the presence of 80% formamide, 400 mM NaCl, and 1 mM EDTA. In each case, 15 µL of the complementary single strands were combined and heated for 10 minutes at 85 °C The mixtures were subsequently incubated overnight at 50 °C and cooled to room temperature.

[0038] Only approximately equimolar amounts of the two single strands were employed in the hybridization. As a result, the dsRNA preparations contained single-stranded RNA (ssRNA) as a contaminant. In order to remove these ssRNA contaminants, the mixtures were treated, after hybridization, with the single-strand-specific ribonucleases, bovine pancreatic RNase A and *Aspergillus oryzae* RNase T1. RNase A is an endoribonuclease specific for pyrimidines. RNase T1 is an endoribonuclease that preferentially cleaves at the 3' side of guanosines. dsRNA is not a substrate for these ribonucleases. For the RNase treatment, 1.2 µL of RNase A at a concentration of 10 mg/mL and 2 µL of RNase T1 at a concentration of 290 µg/mL were added to the mixtures in 300 µL of Tris, pH 7.4, 300 mM NaCl, and 5 mM EDTA. The mixtures were incubated for 1.5 hours at 30 °C Thereupon, the RNases were denatured by addition of 5 µL of

proteinase K at a concentration of 20 mg/mL and 10 µL of 20% SDS and incubation for 30 minutes at 37 °C. The dsRNA was purified by phenol extraction and precipitated with ethanol. To be able to verify the completeness of the RNase digestion, two control mixtures were treated with ssRNA analogously to the hybridization mixtures.

[0039] The dried pellet was taken up in 15 µL of TE buffer, pH 6.5, and subjected to native polyacrylamide gel electrophoresis on an 8% gel. The acrylamide gel was subsequently stained in an ethidium bromide solution and washed in a water bath. Figure 2 shows the RNA visualized in a UV transilluminator. The *sense* RNA applied to lane 1 and the *antisense* RNA applied to lane 2 showed a different migration behavior under the chosen conditions than the dsRNA of the hybridization mixture applied to lane 3. The RNase-treated *sense* RNA and *antisense* RNA applied to lanes 4 and 5, respectively, produced no visible band. This shows that the single-stranded RNAs had been degraded completely. The RNase-treated dsRNA of the hybridization reaction which had been applied to lane 6 is resistant to RNase treatment. The band that migrates faster in the native gel in comparison with the dsRNA applied to lane 3 results from dsRNA free of ssRNA. In addition to the dominant main band, weaker, more rapidly migrating bands are observed after the RNase treatment.

In vitro Transcription Test with Human Nuclear Extract:

[0040] Using the HeLaScribe® Nuclear Extract *in vitro* transcription kit from Promega, Madison, USA, the transcription efficiency of the abovementioned DNA fragment which is present in plasmid pCMV1200 and homologous to the "positive control DNA" was determined in the presence of the dsRNA (dsRNA-CMV5) with sequence homology. Also, the effect of the dsRNA without sequence homology, which corresponds to the yellow fluorescent protein (YFP) gene (dsRNA-YFP), was studied. This dsRNA had been generated analogously to the dsRNA with sequence homology. The sequence of a strand of this dsRNA can be found in sequence ID No. 5. Plasmid pCMV1200 was used as the template for the *run-off* transcription. It carries the "immediate early" cytomegalovirus promoter which is recognized by eukaryotic RNA polymerase II, and a transcribable DNA fragment. Transcription was carried out by means of the HeLa nuclear extract, which contains all proteins necessary for transcription. A radiolabeled transcript was obtained by addition of [•-^{32}P]rGTP to the transcription mixture. The [•-^{32}P]rGTP used had a specific activity of 400 Ci/mmol, 10 mCi/mL. 3 mM $MgCl_2$, in each case 400 µM rATP, rCTP, rUTP, 16 µM rGTP, 0.4 µM [•-^{32}P]rGTP, and depending on the experiment 1 fmol of linearized plasmid DNA and various amounts of dsRNA in transcription buffer were employed

per mixture. Each mixture was made up to a volume of 8.5 µL with H_2O. The mixtures were mixed carefully. To start the transcription, 4 U of HeLa nuclear extract in a volume of 4 µL were added and incubated for 60 minutes at 30 °C The reaction was stopped by addition of 87.5 µL of stop mix warmed to 30 °C To remove the proteins, the mixtures were treated with 100 µL of phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v) saturated with TE buffer, pH 5.0, and the mixtures were mixed vigorously for 1 minute. For phase separation, the mixtures were spun for approximately 1 minute at 12,000 rpm and the top phase was transferred to a fresh reaction vessel. 250 µL of ethanol was added to each mixture. The mixtures were mixed thoroughly and incubated for at least 15 minutes on dry ice/methanol. To precipitate the RNA, the mixtures were centrifuged for 20 minutes at 12,000 rpm and 40 °C. The supernatant was discarded. The pellet was dried in vacuo for 15 minutes and resuspended in 10 µL of H_2O. Each mixture was treated with 10 µL of denaturing loading buffer. The free GTP was separated from the formed transcript by means of denaturing polyacrylamide gel electrophoresis on an 8% gel with 7 M urea. The RNA transcripts formed upon transcription with HeLa nuclear extract, in denaturing loading buffer, were heated for 10 minutes at 90 °C, and 10-µL aliquots were applied immediately to the freshly washed sample pockets. The electrophoresis was run at 40 mA. The amount of the radioactive ssRNA formed upon transcription was analyzed after the electrophoresis with the aid of an *Instant Imager*.

[0041] Figure 3 shows the radioactive RNA from a representative test, visualized by means of the *Instant Imager*. Samples obtained from the following transcription mixtures were applied:

Lane 1:	without template DNA, without dsRNA;
Lane 2:	50 ng of template DNA, without dsRNA;
Lane 3:	50 ng of template DNA, 0.5 µg of dsRNA-YFP;
Lane 4:	50 ng of template DNA, 1.5 µg of dsRNA-YFP;
Lane 5:	50 ng of template DNA, 3 µg of dsRNA-YFP;
Lane 6:	50 ng of template DNA, 5 µg of dsRNA-YFP;
Lane 7:	without template DNA, 1.5 of dsRNA-YFP;
Lane 8:	50 ng of template DNA, without dsRNA;
Lane 9:	50 ng of template DNA, 0.5 µg of dsRNA-CMV5;
Lane 10:	50 ng of template DNA, 1.5 µg of dsRNA-CMV5;
Lane 11:	50 ng of template DNA, 3 µg of dsRNA-CMV5;
Lane 12:	50 ng of template DNA, 5 µg of dsRNA-CMV5;

[0042] It emerged that the amount of transcript was reduced markedly in the presence of dsRNA with sequence homology in comparison with the control mixture without dsRNA and with the mixtures with dsRNA-YFP without sequence homology. The positive control in lane 2 shows that a radioactive transcript was formed in the *in vitro* transcription with HeLa nuclear extract. The mixture is used for comparison with the transcription mixtures incubated in the presence of dsRNA. Lanes 3 to 6 show that the addition of non-sequence-specific dsRNA-YFP had no effect on the amount of transcript formed. Lanes 9 to 12 show that the addition of an amount between 1.5 and 3 µg of sequence-specific dsRNA-CMV5 leads to a reduction in the amount of transcript formed. In order to rule out that the observed effects are based not on the dsRNA but on any contamination that might have been carried along accidentally during the preparation of the dsRNA, a further control was carried out. Single-stranded RNA was transcribed as described above and subsequently subjected to the RNase treatment. It was demonstrated by means of native polyacrylamide gel electrophoresis that the ssRNA had been degraded completely. This mixture was subjected to phenol extraction and ethanol precipitation and subsequently taken up in PE buffer, as were the hybridization mixtures. This gave a sample that contained no RNA but had been treated with the same enzymes and buffers as the dsRNA. Lane 8 shows that the addition of this sample had no effect on transcription. The reduction of the transcript upon addition of sequence-specific dsRNA can therefore be ascribed unequivocally to the dsRNA itself. The reduction in the amount of transcript of a gene in the presence of dsRNA in a human transcription system indicates inhibition of the expression of the gene in question. This effect can be attributed to a novel mechanism caused by the dsRNA.

Exemplary Embodiment 2:

[0043] The test system used for these *in vivo* experiments was the murine fibroblast cell line NIH3T3, ATCC CRL-1658. The YFP gene was introduced into the nuclei with the aid of microinjection. Expression of YFP was studied under the effect of simultaneously cotransfected dsRNA with sequence homology. This dsRNA-YFP shows homology to the 5' region of the YFP gene over a length of 315 bp. The nucleotide sequence of a strand of the dsRNA-YFP is shown in sequence ID No. 5. Evaluation under the fluorescence microscope was carried out 3 hours after injection with reference to the greenish-yellow fluorescence of the YFP formed.

Construction of the Template Plasmid and Preparation of the dsRNA:

[0044] A plasmid was constructed following the same principle as described in Exemplary Embodiment 1 to act as a template for the preparation of the YFP-dsRNA by means of T7 and SP6 *in vitro* transcription. Using the primer *Eco*_T7_YFP as shown in sequence ID No. 6 and *Bam*_SP6_YFP as shown in sequence ID No. 7, the desired gene fragment was amplified by PCR and used analogously to the above description for preparing the dsRNA. The dsRNA-YFP obtained is identical to the dsRNA used in Exemplary Embodiment 1 as the non-sequence-specific control.

[0045] A dsRNA linked chemically at the 3' end of the RNA, as shown in sequence ID No. 8, to the 5' end of the complementary RNA via a C18 linker group was prepared (L-dsRNA). To this end, synthons modified by disulfide bridges were used. The 3'-terminal synthon is bound to the solid support via the 3' carbon to an aliphatic linker group via a disulfide bridge. In the 5'-terminal synthon of the complementary oligoribonucleotide which is complementary to the 3'-terminal synthon of the one oligoribonucleotide, the 5'-trityl protecting group is bound via a further aliphatic linker and a disulfide bridge. Following synthesis of the two single strands, removal of the protecting groups, and hybridization of the complementary oligoribonucleotides, the forming thiol groups are spatially close to one another. The single strands are linked to each other by oxidation via their aliphatic linkers and a disulfide bridge. This is followed by purification with the aid of HPLC.

Preparation of the Cell Cultures:

[0046] The cells were incubated in DMEM, supplemented with 4.5 g/L of glucose and 10% fetal bovine serum, in culture dishes at 37 °C under a 7.5% CO_2 atmosphere and passaged before reaching confluence. The cells were detached with trypsin/EDTA. To prepare the microinjection, the cells were transferred to petri dishes and incubated further until microcolonies formed.

Microinjection:

[0047] For the microinjection, the culture dishes were removed from the incubator for approximately 10 minutes. Approximately 50 nuclei were injected singly per mixture within a marked area using the AIS microinjection system from Carl Zeiss, Gottingen, Germany. The cells were subsequently incubated for 3 more hours. For the microinjection, borosilicate glass

capillaries from Hilgenberg GmbH, Malsfeld, Germany, with a tip diameter of less than 0.5 µm were prepared. The microinjection was carried out using a micromanipulator from Narishige Scientific Instrument Lab., Tokyo, Japan. The injection time was 0.8 seconds and the pressure was approximately 100 hPa. The transfection was carried out using the plasmid pCDNA-YFP, which contains an approximately 800 bp *Bam*HI/*Eco*RI fragment with the YFP gene in vector pcDNA3. The samples injected into the nuclei contained 0.01 µg/L of pCDNA-YFP and Texas Red coupled to dextran-70000 in 14 mM NaCl, 3 mM KCl, and 10 mM KPO_4, pH 7.5. Approximately 100 pL of RNA with a concentration of 1 µM or, in the case of L-dsRNA, 375 µM were additionally added.

[0048] The cells were examined using a fluorescence microscope with excitation with the light of the excitation wavelength of Texas Red, 568 nm, or of YFP, 488 nm. Individual cells were documented by means of a digital camera. Figures 4a–e show the result for NIH3T3 cells. In the cells shown in Fig. 4a, *sense*-YFP-ssRNA has been injected, in Fig. 4b *antisense*-YFP-ssRNA, in Fig. 4c dsRNA-YFP, in Fig. 4d no RNA, and in Fig. 4e L-dsRNA.

[0049] The field on the left shows in each case the fluorescence of cells with excitation at 568 nm. The fluorescence of the same cells at an excitation of 488 nm is seen on the right. The Texas Red fluorescence of all the shown cells demonstrates that the injection solution had been injected successfully into the nuclei and that cells with successful hits were still alive after 3 hours. Dead cells no longer showed Texas Red fluorescence.

[0050] The right fields of each of Figs. 4a and 4b show that YFP expression was not visibly inhibited when the single-stranded RNA was injected into the nuclei. The right field of Fig. 4c shows cells whose YFP fluorescence was no longer detectable after the injection of dsRNA-YFP. Figure 4d shows cells into which no RNA had been injected, as control. The cell shown in Fig. 4e shows YFP fluorescence which can no longer be detected owing to the injection of L-dsRNA, which has regions with sequence homology to the YFP gene. This result demonstrates that even shorter dsRNAs can be used for specifically inhibiting gene expression in mammals when the double strands are stabilized by chemically linking the single strands.

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SEQUENCE LISTING

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Claims

1. Method for inhibiting the expression of a given target gene in a cell in vitro, where an oligoribonucleotide with double-stranded structure (dsRNA) formed by two separate RNA single strands is introduced into the cell, where one strand of the dsRNA has a region which is complementary to the target gene,
characterized in that
the complementary region has less than 25 successive nucleotide pairs.

2. Method according to claim 1, where the dsRNA is enclosed by micellar structures, preferably by liposomes.

3. Method according to either of the preceding claims, where the dsRNA is enclosed by natural viral capsids or by chemically or enzymatically produced artificial capsids or structures derived therefrom.

4. Method according to one of the preceding claims, where the target gene is expressed in eukaryotic cells.

5. Method according to one of the preceding claims, where the target gene is selected from the following group: oncogene, cytokin gene, Id-protein gene, development gene, prion gene.

6. Method according to one of the preceding claims, where the target gene is expressed in pathogenic organisms, preferably in plasmodia.

7. Method according to one of the preceding claims, where the target gene is part of a virus or viroid.

8. Method according to claim 7, where the virus is a virus or viroid which is pathogenic for humans.

9. Method according to claim 7, where the virus or viroid is a virus or viroid which is pathogenic for animals or phytopathogenic.

10. Method according to one of the preceding claims, where segments of the dsRNA are in double-stranded form.

11. Method according to one of the preceding claims, where the ends of the dsRNA are modified in order to counteract degradation in the cell or dissociation into the single strands.

12. Method according to one of the preceding claims, where the cohesion of the double-stranded structure, which is caused by the complementary nucleotide pairs, is increased by at least one, preferably two, further chemical linkage (s).

13. Method according to one of the preceding claims, where the chemical linkage is formed by a covalent or ionic bond, a hydrogen bond, hydrophobic interactions, preferably van-der-Waals or stacking interactions, or by metal-ion coordination.

14. Method according to one of the preceding claims, where the chemical linkage is generated at at least one, preferably both, ends of the double-stranded structure.

15. Method according to one of the preceding claims, where the chemical linkage is formed by means of one or more compound groups, the compound groups preferably being poly(oxyphosphinicooxy-1,3-propanediol) and/or polyethylene glycol chains.

16. Method according to one of the preceding claims, where the chemical linkage is formed by purine analogs used in the double-stranded structure in place of purines.

17. Method according to one of the preceding claims, where the chemical linkage is formed by azabenzene units introduced into the double-stranded structure.

18. Method according to one of the preceding claims, where the chemical linkage is formed by branched nucleotide analogs used in the double-stranded structure in place of nucleotides.

19. Method according to one of the preceding claims, where at least one of the following groups is used for generating the chemical linkage: methylene blue; bifunctional groups, preferably bis(2-chloroethyl)amine; N-acetyl-N'-(p-glyoxylbenzoyl)cystamine; 4-thiouracil; psoralene.

20. Method according to one of the preceding claims, where the chemical linkage is formed by thiophosphoryl groups provided at the ends of the double-stranded structure.

21. Method according to one of the preceding claims, where the chemical linkage at the ends of the double-stranded structure is formed by triple-helix bonds.

22. Method according to one of the preceding claims, where at least one 2'-hydroxyl group of the nucleotides of the dsRNA in the double-stranded structure is replaced by a chemical group, preferably a 2'-amino or a 2'-methyl group.

23. Method according to one of the preceding claims, where at least one nucleotide in at least one strand of the double-stranded structure is a locked nucleotide with a sugar ring which is chemically modified, preferably by a 2'-O, 4'-C-methylene bridge.

24. Method according to one of the preceding claims, where the dsRNA is bound to, associated with or surrounded by, at least one viral coat protein which originates from a virus, is derived therefrom or has been prepared synthetically.

25. Method according to one of the preceding claims, where the coat protein is derived from polyomavirus.

26. Method according to one of the preceding claims, where the coat protein contains the polyomavirus virus protein 1 (VP1) and/or virus protein 2 (VP2).

27. Method according to one of the preceding claims, where, when a capsid or capsid-type structure is formed from the coat protein, one side faces the interior of the capsid or capsid-type structure.

28. Method according to one of the preceding claims, where one strand of the dsRNA is complementary to the primary or processed RNA transcript of the target gene.

29. Method according to one of the preceding claims, where the cell is a vertebrate cell or a human cell.

30. Method according to one of the preceding claims, where at least two dsRNAs which differ from each other are introduced into the cell, where at least segments of one strand of each dsRNA are complementary to in each case one of at least two different target genes.

31. Method according to one of the preceding claims, where one of the target genes is the PKR gene.

32. Medicament with at least one oligoribonucleotide with double-stranded structure (dsRNA) formed by two separate RNA single strands for inhibiting the expression of a given target gene, where one strand of the dsRNA has a region which is complementary to the target gene,
characterized in that
the complementary region has less than 25 successive nucleotide pairs.

33. Medicament according to claim 32, where the dsRNA is enclosed by micellar structures, preferably by liposomes.

34. Medicament according to either of claims 32 or 33, where the dsRNA is enclosed by natural viral capsids or by chemically or enzymatically produced artificial capsids or structures derived therefrom.

35. Medicament according to one of claims 32 to 34, where the target gene can be expressed in eukaryotic cells.

36. Medicament according to one of claims 32 to 35, where the target gene is selected from the following group: oncogene, cytokin gene, id-protein gene, development gene, prion gene.

37. Medicament according to one of claims 32 to 36, where the target gene can be expressed in pathogenic organisms, preferably in plasmodia.

38. Medicament according to one of claims 32 to 37, where the target gene is part of a virus or viroid.

39. Medicament according to claim 38, where the virus is a virus or viroid which is pathogenic for humans.

40. Medicament according to claim 38, where the virus or viroid is a virus or viroid which is pathogenic for animals.

41. Medicament according to one of claims 32 to 40, where segments of the dsRNA are in double-stranded form.

42. Medicament according to one of claims 32 to 40, where the ends of the dsRNA are modified in order to counteract degradation in the cell or dissociation into the single strands.

43. Medicament according to one of claims 32 to 42, where the cohesion of the double-stranded structure, which is caused by the complementary nucleotide pairs, is increased by at least one, preferably two, further chemical linkage (s).

44. Medicament according to one of claims 32 to 43, where the chemical linkage is formed by a covalent or ionic bond, a hydrogen bond, hydrophobic interactions, preferably van-der-Waals or stacking interactions, or by metal-ion coordination.

45. Medicament according to one of claims 32 to 44, where the chemical linkage is generated at at least one, preferably both, ends of the double-stranded structure.

46. Medicament according to one of claims 32 to 45, where the chemical linkage is formed by means of one or more compound groups, the compound groups preferably being poly(oxyphosphinicooxy-1,3-propanediol) and/or poly-ethylene glycol chains.

47. Medicament according to one of claims 32 to 46, where the chemical linkage is formed by purine analogs used in the double-stranded structure in place of purines.

48. Medicament according to one of claims 32 to 47, where the chemical linkage is formed by azabenzene units inserted into the double-stranded structure.

49. Medicament according to one of claims 32 to 48, where the chemical linkage is formed by branched nucleotide analogs used in the double-stranded structure in place of nucleotides.

50. Medicament according to one of claims 32 to 49, where at least one of the following groups is used for generating the chemical linkage: methylene blue; bifunctional groups, preferably bis(2-chloroethyl)amine; N-acetyl-N'-(p-glyoxylbenzoyl) - cystamine; 4-thiouracil; psoralene.

51. Medicament according to one of claims 32 to 50, where the chemical linkage is formed by thiophosphoryl groups provided at the ends of the double-stranded structure.

52. Medicament according to one of claims 32 to 51, where the chemical linkage are [sic] triple-helix bonds provided at the ends of the double-stranded structure.

53. Medicament according to one of claims 32 to 52, where at least one 2'-hydroxyl group of the nucleotides of the dsRNA in the double-stranded structure is replaced by a chemical group, preferably a 2'-amino or a 2'-methyl group.

54. Medicament according to one of claims 32 to 53, where at least one nucleotide in at least one strand of the double-stranded structure is a locked nucleotide with a sugar ring which is chemically modified, preferably by a 2'-O, 4'-C-methylene bridge.

55. Medicament according to one of claims 32 to 54, where the dsRNA is bound to, associated with or surrounded by, at least one viral coat protein which originates from a virus, is derived therefrom or has been prepared synthetically.

56. Medicament according to one of claims 32 to 55, where the coat protein is derived from the polyomavirus.

57. Medicament according to one of claims 32 to 56, where the coat protein contains the polyomavirus virus protein 1 (VP1) and/or virus protein 2 (VP2).

58. Medicament according to one of claims 32 to 57, where, when a capsid or capsid-type structure is formed from the coat protein, one side faces the interior of the capsid or capsid-type structure.

59. Medicament according to one of claims 32 to 58, where one strand of the dsRNA is complementary to the primary or processed RNA transcript of the target gene.

60. Medicament according to one of claims 32 to 59, where the cell is a vertebrate cell or a human cell.

61. Medicament according to one of claims 32 to 60, where at least two dsRNAs which differ from each other are contained in the medicament, where at least segments of one strand of each dsRNA are complementary to in each case one of at least two different target genes.

62. Medicament according to claim 61, where one of the target genes is the PKR gene.

63. Active ingredient with at least one oligoribonucleotide with double-stranded structure (dsRNA) formed by two separate RNA single strands for inhibiting the expression of a given target gene, where one strand of the dsRNA has a region which is complementary to the target gene, and where the target gene is part of a phytopathogenic virus or viroid,
characterized in that
the complementary region has less than 25 successive nucleotide pairs.

64. Active ingredient according to claim 63, where the target gene can be expressed in eukaryotic cells.

65. Active ingredient according to claim 63 or 64, where segments of the dsRNA are in double-stranded form.

66. Active ingredient according to one of claims 63 to 65, where the ends of the dsRNA are modified in order to counteract degradation in the cell or dissociation into the single strands.

67. Active ingredient according to one of claims 63 to 66, where the cohesion of the double-stranded structure, which is caused by the complementary nucleotide pairs, is increased by at least one, preferably two, further chemical

68. Active ingredient according to one of claims 63 to 67, where the chemical linkage is formed by a covalent or ionic bond, a hydrogen bond, hydrophobic interactions, preferably van-der-Waals or stacking interactions, or by metal-ion coordination.

69. Active ingredient according to one of claims 63 to 68, where the chemical linkage is generated at at least one, preferably both, ends of the double-stranded structure.

70. Active ingredient according to one of claims 63 to 69, where the chemical linkage is formed by means of one or more compound groups, the compound groups preferably being poly(oxyphosphinicooxy-1,3-propanediol) and/or polyethylene glycol chains.

71. Active ingredient according to one of claims 63 to 70, where the chemical linkage is formed by purine analogs used in the double-stranded structure in place of purines.

72. Active ingredient according to one of claims 63 to 71, where the chemical linkage is formed by azabenzene units inserted into the double-stranded structure.

73. Active ingredient according to one of claims 63 to 72, where the chemical linkage is formed by branched nucleotide analogs used in the double-stranded structure in place of nucleotides.

74. Active ingredient according to one of claims 63 to 73, where at least one of the following groups is used for generating the chemical linkage: methylene blue; bifunctional groups, preferably bis(2-chloroethyl)amine; N-acetyl-N'-(p-glyoxylbenzoyl)cystamine; 4-thiouracil; psoralene.

75. Active ingredient according to one of claims 63 to 74, where the chemical linkage is formed by thiophosphoryl groups provided at the ends of the double-stranded structure.

76. Active ingredient according to one of claims 63 to 75, where the chemical linkage are triple-helix bonds provided at the ends of the double-stranded structure.

77. Active ingredient according to one of claims 63 to 76, where at least one 2'-hydroxyl group of the nucleotides of the dsRNA in the double-stranded structure is replaced by a chemical group, preferably a 2'-amino or a 2'-methyl group.

78. Active ingredient according to one of claims 63 to 77, where at least one nucleotides at least one strand of the double-stranded structure is a locked nucleotide with a sugar ring which is chemically modified, preferably by a 2'-O, 4'-C-methylene bridge.

79. Active ingredient according to one of claims 63 to 78, where one strand of the dsRNA is complementary to the primary or processed RNA transcript of the target gene.

80. Active ingredient according to one of claims 63 to 79, where at least two dsRNAs which differ from each other are contained in the active ingredient, where at least segments of one strand of each dsRNA are complementary to in each case one of at least two different target genes.

81. Use of an oligoribonucleotide with double-stranded structure (dsRNA) formed by two separate RNA single strands for preparing a medicament or active ingredient for inhibiting the expression of a given target gene, where one strand of the dsRNA has a region which is complementary to the target gene,
characterized in that
the complementary region has less than 25 successive nucleotide pairs.

82. Use according to claim 81, where the dsENA is enclosed by micellar structures, preferably by liposomes.

83. Use according to either of claims 81 or 82, where the dsRNA is enclosed by natural viral capsids or by chemically or enzymatically produced artificial capsids or structures derived therefrom.

84. Use according to one of claims 81 to 83, where the target gene can be expressed in eukaryotic cells.

85. Use according to one of claims 81 to 84, where the target gene is selected from the following group: oncogene, cytokin gene, Id-protein gene, development gene, prion gene.

86. Use according to one of claims 81 to 85, where the target gene can be expressed in pathogenic organisms, preferably in plasmodia.

87. Use according to one of claims 81 to 86, where the target gene is part of a virus or viroid.

88. Use according to claim 87, where the virus is a virus or viroid which is pathogenic for humans.

89. Use according to claim 87, where the virus or viroid is a virus or viroid which is pathogenic for animals or phytopathogenic.

90. Use according to one of claims 81 to 89, where segments of the dsRNA are in double-stranded form.

91. Use according to one of claims 81 to 90, where the ends of the dsRNA are modified in order to counteract degradation in the cell or dissociation into the single strands.

92. Use according to one of claims 81 to 91, where the cohesion of the double-stranded structure, which is caused by the complementary nucleotide pairs, is increased by at least one, preferably two, further chemical linkage(s).

93. Use according to one of claims 81 to 92, where the chemical linkage is formed by a covalent or ionic bond, a hydrogen bond, hydrophobic interactions, preferably van-der-Waals or stacking interactions, or by metal-ion coordination.

94. Use according to one of claims 81 to 93, where the chemical linkage is generated at at least one, preferably both, ends of the double-stranded structure.

95. Use according to one of claims 81 to 94, where the chemical linkage is formed by means of one or more compound groups, the compound groups preferably being poly(oxyphosphinicooxy-1,3-propanediol) and/or polyethylene glycol chains.

96. Use according to one of claims 81 to 95, where the chemical linkage is formed by purine analogs used in the double-stranded structure in place of purines.

97. Use according to one of claims 81 to 96, where the chemical linkage is formed by azabenzene units introduced into the double-stranded structure.

98. Use according to one of claims 81 to 97, where the chemical linkage is formed by branched nucleotide analogs used in the double-stranded structure in place of nucleotides.

99. Use according to one of claims 81 to 98, where at least one of the following groups is used for generating the chemical linkage: methylene blue; bifunctional groups, preferably bis(2-chloroethyl)amine; N-acetyl-N'-(p-glyoxylbenzoyl)-cystamine; 4-thiouracil; psoralene.

100. Use according to one of claims 81 to 99, where the chemical linkage is formed by thiophosphoryl groups attached to the ends of the double-stranded structure.

101. Use according to one of claims 81 to 100, where the chemical linkage at the ends of the double-stranded structure is formed by triple-helix bonds.

102. Use according to one of claims 81 to 101, where at least one 2'-hydroxyl group of the nucleotides of the dsRNA in the double-stranded structure is replaced by a chemical group, preferably a 2'-amino or a 2'-methyl group.

103. Use according to one of claims 81 to 102, where at least one nucleotide in at least one strand of the double-stranded structure is a locked nucleotide with a sugar ring which is chemically modified, preferably by a 2'-O, 4'-C-

methylene bridge.

104. Use according to one of claims 81 to 103, where the dsRNA is bound to, associated with or surrounded by, at least one viral coat protein which originates from a virus, is derived therefrom or has been prepared synthetically.

105. Use according to one of claims 81 to 104, where the coat protein is derived from polyomavirus.

106. Use according to one of claims 81 to 105, where the coat protein contains the polyomavirus virus protein 1 (VP1) and/or virus protein 2 (VP2).

107. Use according to one of claims 81 to 106, where, when a capsid or capsid-type structure is formed from the coat protein, one side faces the interior of the capsid or capsid-type structure.

108. Use according to one of claims 81 to 107, where one strand of the dsRNA is complementary to the primary or processed RNA transcript of the target gene.

109. Use according to one of claims 81 to 108, where the cell is a vertebrate cell or a human cell.

110. Use according to one of claims 81 to 109, where at least two dsRNAs which differ from each other are used, where at least segments of one strand of each dsRNA are complementary to in each case one of at least two different target genes.

111. Use according to claim 110, where one of the target genes is the PKR gene.

112. Use according to one of claims 81 to 111, where the medicament is injectable into the bloodstream or into the interstitium of the organism to undergo therapy.

113. Use according to one of claims 81 to 112, where the dsRNA is taken up into bacteria or microorganisms.

114. Use of a vector for coding at least one oligoribonucleotide with double-stranded structure (dsRNA) formed by two separate RNA single strands for preparing a medicament or active ingredient for inhibiting the expression of a given target gene, where one strand of the dsRNA has a region which is complementary to the target gene, **characterized in that**
the complementary region has less than 25 successive nucleotide pairs.

115. Use according to claim 114, where the target gene can be expressed in eukaryotic cells.

116. Use according to claim 114 or 115, where the target gene is selected from the following group: oncogene, cytokin gene, Id-protein gene, development gene, prion gene.

117. Use according to one of claims 114 to 116, where the target gene can be expressed in pathogenic organisms, preferably in plasmodia.

118. Use according to one of claims 114 to 117, where the target gene is part of a virus or viroid.

119. Use according to claim 118, where the virus is a virus or viroid which is pathogenic for humans.

120. Use according to claim 118, where the virus or viroid is a virus or viroid which is pathogenic for animals or phytopathogenic.

121. Use according to one of claims 114 to 120, where segments of the dsRNA are in double-stranded form.

122. Use according to one of claims 114 to 121, where one strand of the dsRNA is complementary to the primary or processed RNA transcript of the target gene.

123. Use according to one of claims 114 to 122, where the cell is a vertebrate cell or a human cell.

124. Use according to one of claims 114 to 123, where at least two dsRNAs which differ from each other are used,

where at least segments of one strand of each dsRNA are complementary to in each case one of at least two different target genes.

125. Use according to claim 125, where one of the target genes is the PKR gene.

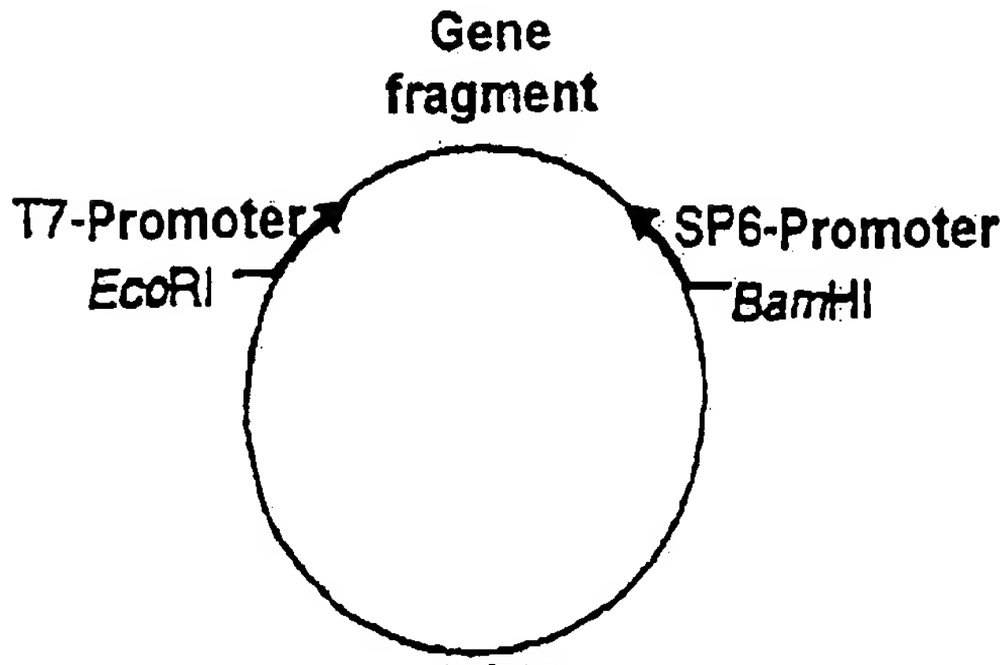
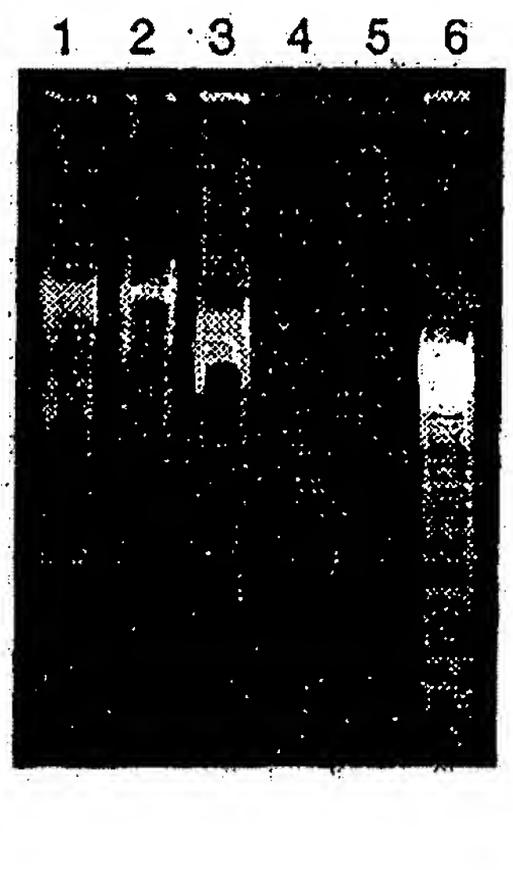


Fig. 1



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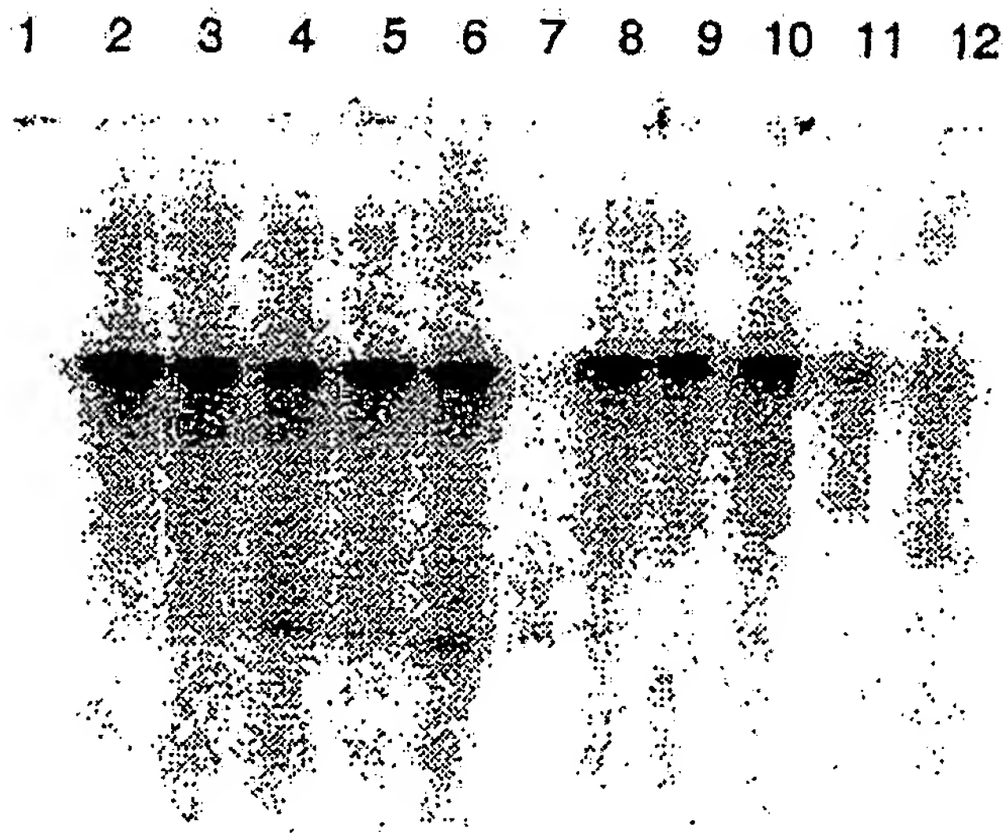

Fig. 3

Fig. 4 a

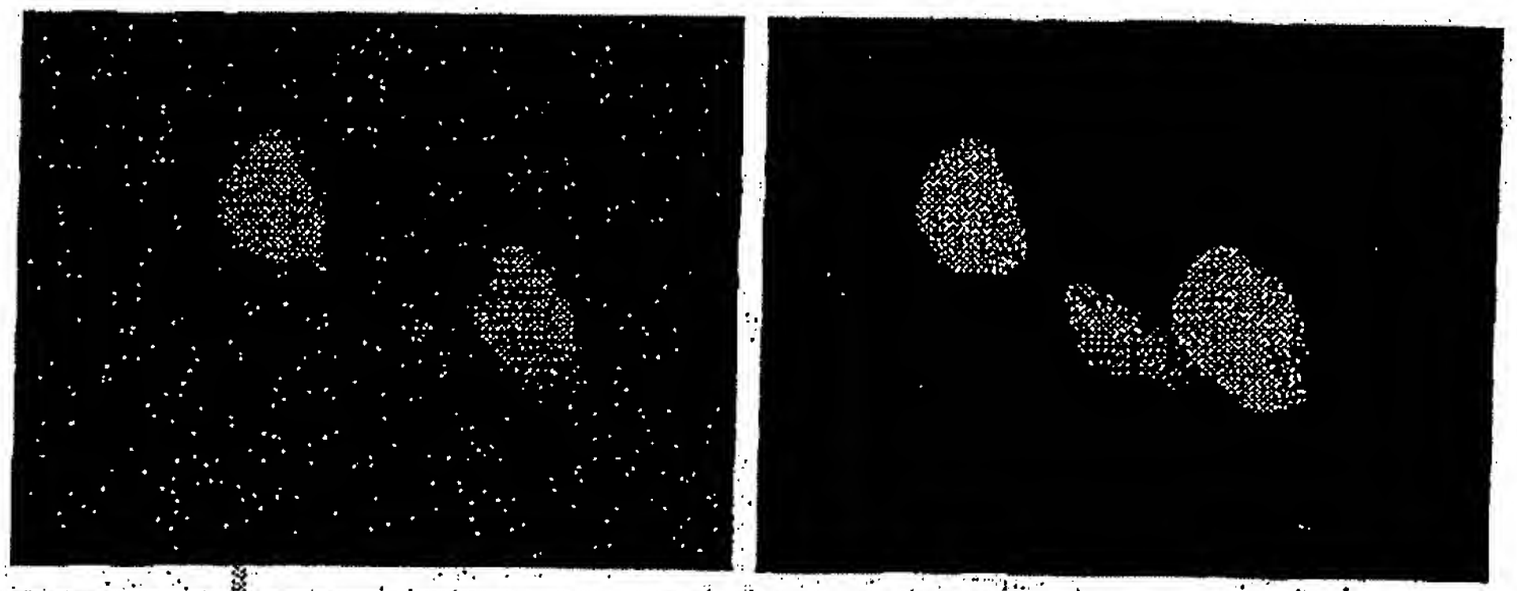

Fig. 4 b

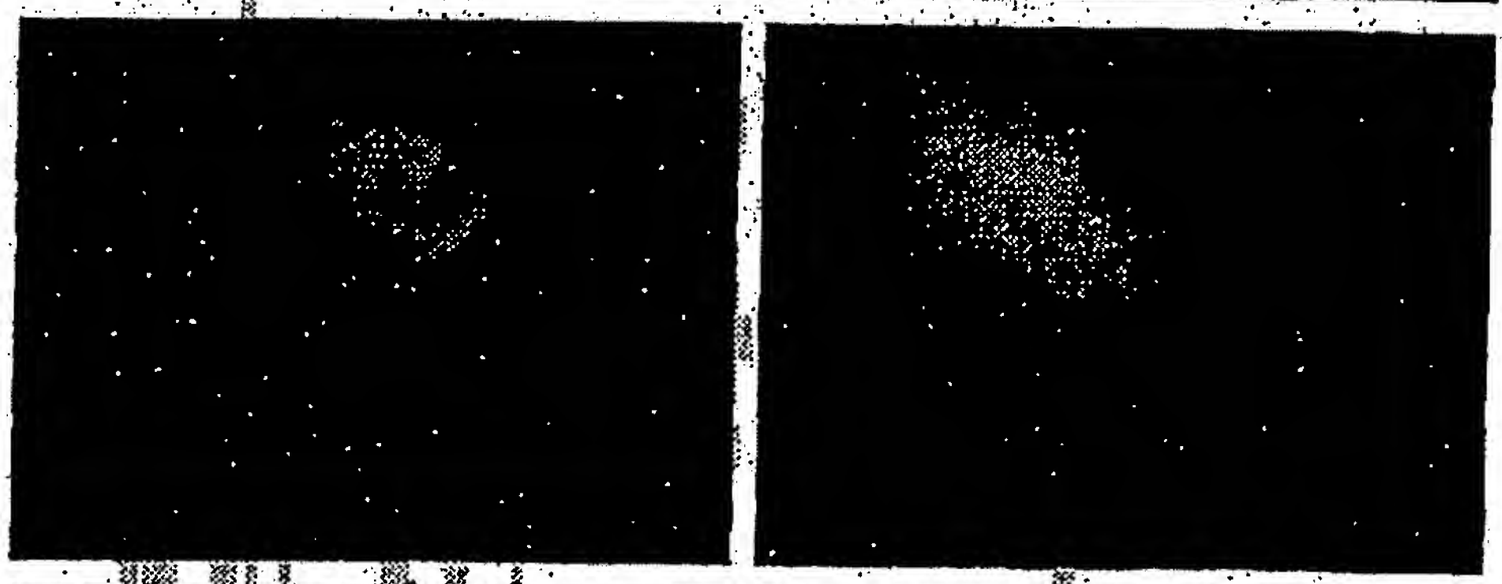

Fig. 4 c

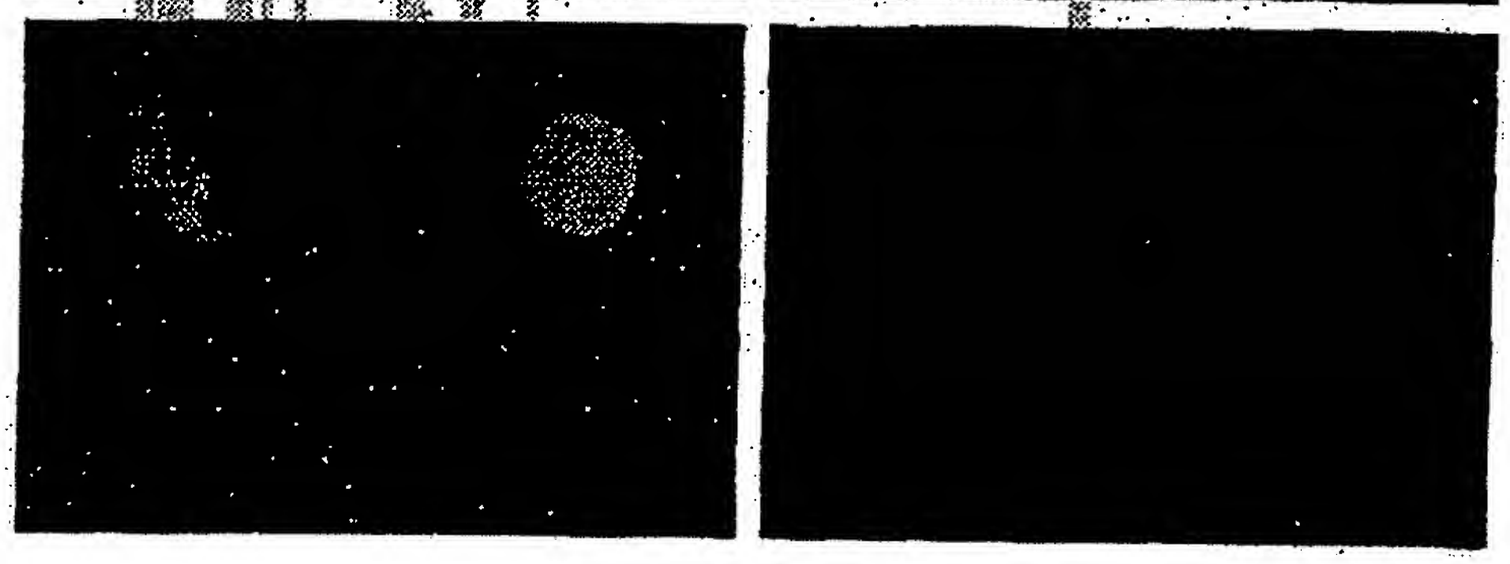

Fig. 4 d

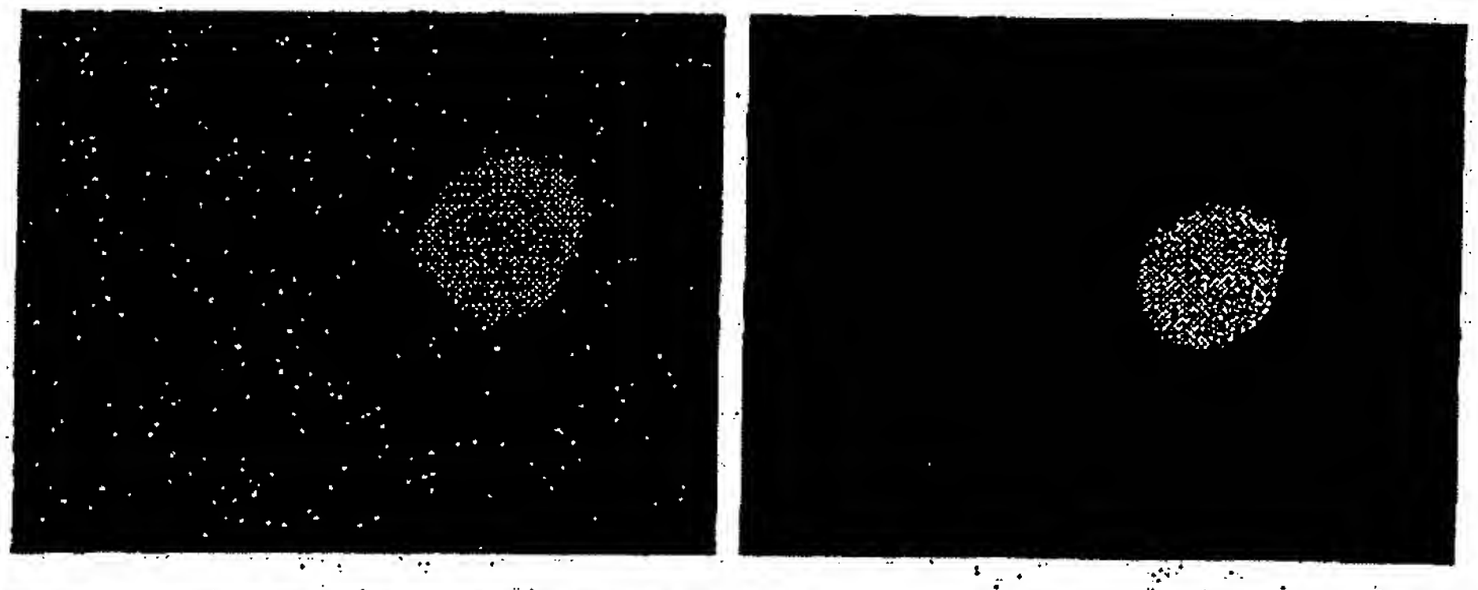

Fig. 4 e